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REVIEW

Gene delivery systems for use in gene therapy: an overview of quality assurance and safety issues

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The development of safe and effective agents for gene therapy is founded on three main principles; careful choice and design of vectors, assessment of vector safety under GLP and production of the vector stocks under GMP. The first ensures the safe and appropriate contained delivery and expression of the required gene to the recipient of the therapy. GLP provides fully documented studies of potency, efficacy and safety of the product while the production of clinical grade agents under GMP is essential.

Keywords: *regulatory issues; GMP production; validation*

Introduction

Advances in recombinant DNA-based science have led to the ability to characterize disease at the level of specific genes while the technology to diagnose either acquired or inherited disease has developed in parallel. Similarly, potentially therapeutic DNA-based products can now be engineered to correct these defects. Thus the basic tenet of gene-based therapies is the effective delivery of therapeutic genes to mammalian cells and subsequent expression of the gene in an appropriate manner.

Initially, the main thrust of gene therapy was to correct heritable genetic lesions by replacement or control of the function of the affected genes. However, gene delivery has many potential beneficial applications, including the treatment of genetic disorders, malignancies, viral infections and for cell tagging.[1] The regulatory considerations applicable to these novel agents are discussed below.

Gene delivery systems

The initial step in gene therapy is the transduction (by viral vectors) or transfection (by DNA-mediated systems) of appropriate target cells. Target specificity, efficiency of delivery and expression of the therapeutic gene is paramount in the design of any gene therapy protocol. The use of non-viral DNA delivery systems for gene therapy has a number of features that make them attractive alternatives to viral systems, including ease of manipulation, safety and stability on storage and thus a number of approaches have been developed for delivery of DNA to cells.

The choice of vector systems for delivery and expression of genes in cells either *in vitro* or *in vivo* depends on a number of factors:

- The target cell or tissue;
- A requirement for permanent alterations to the genome;
- Levels of expression required;
- Means of delivery and dose regimen;
- Use as vaccines;
- Safety issues.

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Viral delivery systems

There is no single viral vector or derivative that can provide a suitably flexible approach to the many applications of gene therapy described previously. Many viruses can be adapted for use as vectors for gene therapy allowing one to design vectors from viruses with properties useful in particular situations. It is beyond the scope of this article to discuss the design and features of these vectors in detail and these are the subject of a number of reviews.[1–5] The requirement for flexibility is reflected in the wide range of viruses used in vector construction to date which are summarized in Table 1.

DNA delivery systems

Non-viral DNA delivery systems rely on cellular mechanisms to import DNA into the cell and to transport the DNA to the nucleus where it is expressed. Thereby, heterologous genes can be transferred directly into tissues *in vivo* with concomitant expression, as has already been demonstrated in muscle cells.[6] There are a number of approaches in current use exploiting the direct delivery of DNA for gene therapy and vaccination. All of these methods apparently result in transient expression of the gene of interest and integration or stable expression of these constructs has not been observed to date.

The potential advantages of using direct delivery of DNA over the use of viral vectors are:

- There is less of a limitation to the size of DNA which can be used and up to 48-kb fragments have been successfully transferred to cells.[7]
- The DNA can be easily manipulated without the limitations of essential viral sequences required for packaging and replication.

Table 1 Viral delivery systems for gene therapy and vaccines

Virus type	*Integration*	*Tissue specificity*	*Properties*
Retroviruses*	Yes	Yes	Infect only dividing cells[7]
Lentiviruses*	Yes	Yes	Infect stationary phase cells[7]
Adenoviruses*	Sometimes	Yes	Infect non-dividing cells[8]
Herpes viruses*	No	Yes	Useful for cells of central nervous system[9]
EBV			
Herpes simplex			
CMV			
Pseudorabies			
Vaccinia virus	No	No	Wide host range useful as vector in immunization[10]
Poliovirus	No	No	Wide range of cells infected[11]
Sindbis	No	No	Wide host range[12]
Semliki forest virus	No	No	Infect wide variety of cells[13]
Parvovirus	No	Yes	Infect dividing tumour cells preferentially[14]
Adeno-associated virus*	Yes	No	Integrate in non-dividing cells[15]

*These are currently the most advanced gene therapy viral vector systems.

- There are fewer safety considerations for the production of DNA-complexes compared with 'infectious' viral vectors as the constructs are not likely to be infectious.
- There are potentially less problems with immunogenicity.
- The DNA is broken down rapidly in plasma and the half-life of the DNA is shortened.

However, the apparent simplicity of utilizing DNA directly for expression in target muscle cells is misleading and low efficiency of transfection and therefore gene expression has been reported.[8] Although this may not r strict certain therapies or vaccination protocols, increasing the efficiency of transfection is an important consideration.

One major advantage of using viral vectors for gene therapy is that the tissue or cell tropism of the vector will target the gene of interest to the correct place. Such targeting of DNA delivery systems is more problematic but the difficulties of targeting DNA to the appropriate cell type or tissue can be overcome in a number of ways:

- Direct injection of the DNA into tissue leads to physical targeting since DNA has a short half-life in tissue and serum and will not be replicated.
- Complexing DNA with molecules recognized by cell-specific receptors or antibodies recognizing cell antigen can also prove useful.
- Similar to viral systems utilizing a tissue or cell-specific promoter to the coding region required to be expressed results in 'appropriate' expression.

Such regulator sequence-driven specificity is useful but full characterization of such promoters is essential to ensure that other factors do not overcome the specificity of the regulatory sequences.

There are a number of other potential therapies based on chemically synthesized DNA and RNA including oligonucleotides (sense and antisense), peptide DNA, oligoRNA and ribozymes which are beyond the scope of this article.[16,17]

Regulatory concerns

The production of viral vectors, naked DNA or conjugated DNA for gene therapy/vaccine applications will require careful regulation. There are three main areas of potential regulatory concerns to be considered in the design, production and delivery of recombinant gene therapy agents, whether virus or DNA based (Figure 1):

- Environmental issues;
- Pathogenicity of the agent;
- Characterization and quality assurance of the seed banks and vector stocks.

These areas will be considered in more detail here although the application to individual agents will vary.

Environmental issues

The use of infectious recombinant materials including both viral vectors and to a lesser extent naked or conjugated DNA, may be considered to be a deliberate release

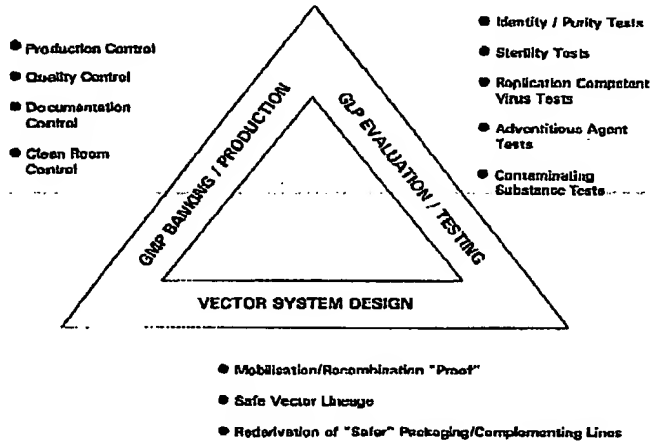

Figure 1 Safety triad.

of genetically modified organisms. Thus, there may be a requirement to comply with international directives on such deliberate releases,[18–20] although in the UK viral vectors for gene therapy are considered by the Gene Therapy Advisory Committee (GTAC)[21] and the Medicines Control Agency (MCA) rather than by the Advisory Committee on the Release of Recombinant Organisms (ACRE).

Deliberate release considerations may not be concerned with the efficacy or validity of the construct but rather with environmental risk assessment. In the European Council Directive,[19] for example, the information required in the notification includes the following:

- A detailed description of the construct;
- Comparative data for both the parental and recombinant virus/DNA including host range, transmission, persistence virulence, stability of constructs, recombination and mutation frequencies.
- Details on the release and the environment of the release. Control of the release and emergency response plans.
- Potential interactions between the GMO and the environment including potential impact on the environment and effects on non-target organisms.
- Monitoring techniques for tracing the recombinant and any potential transfer of genetic material from the recombinant.
- Waste treatment.

Pathogenicity of gene therapy agents

Gene therapy delivery systems themselves can be considered to be pathogens, particularly the viral vectors. There are no general tests which can predict virulence or pathogenicity of recombinant vectors and, therefore, each construct must be considered on an individual basis. In general, data collected from a number of pertinent studies would be ideal and would give a better idea of the pathogenic potential of a recombinant virus.[22]

Several *in vivo* viral gene delivery vectors have been derived from viruses which have been used in extensive immunization programmes including adenovirus, vaccinia virus and poliovirus[4,11,23,24] (Table 1). Therefore, data exist for morbidity and potential side-effects of the parental virus allowing some insight into potential problem areas in a related recombinant vector which can then be addressed by conducting comparative studies.

Comparative studies in immunosuppressed animals have already proved useful in characterizing the pathogenicity of some recombinant viruses.[25] Nevertheless, there are no reliable tests to indicate or predict the efficacy or virulence of recombinant viruses or the expression of the gene products in humans. Pre-clinical *in vivo* testing of the recombinant agents should be useful for evaluating potential problems. The systems used may involve animal models and each therapy will require different model systems. In addition, transgenic animals with appropriate genetic makeup, or immunosuppressed animals carrying xenographs should be useful for these studies.

The design of the vector itself contributes significantly to the safety of a therapy. Many of the gene delivery systems used in gene therapy protocols are promiscuous and will target a wide range of cells and tissue types. Inclusion of tissue-specific promoter/enhancer sequences should limit gene expression to the appropriate cells and circumvent this problem. Control of the levels of gene expression should reduce problems of toxicity and immunogenicity of the products. Incorporation of a suicide gene, eg thymidine kinase in the vector should allow systemic drug treatment of the vector recipient to kill cells harbouring the virus vector in cases where problems with therapy occur or where there is a desire to limit the therapy.

Data from clinical trials are of obvious value in assessment of the therapeutic agent.

Good manufacturing practice (GMP) and good laboratory practice (GLP) standards

The effective characterization and safe controlled production of an agent for medicinal use requires that particular attention is addressed to its design and manufacture. Two codes of practice have been developed to ensure conformity to these requirements: good manufacturing practice (GMP) and good laboratory practice (GLP). The basic principles of these codes of practice is discussed briefly below.

Good manufacturing practice (GMP)

The Medicines Control Agency 1993 Rules and Guidance for Pharmaceutical Manufacturers[26] states: 'The majority of reported defective medicinal products has resulted from human error or carelessness'.

In order to prevent or limit the possibilities of such errors occurring unknowingly a system of controls with emphasis on hierarchical testing and documentation, GMP, has been developed for the manufacture of medicinal products including biologicals.

GMP is 'the part of quality assurance which ensures that medicinal products are consistently produced and controlled to the quality standards appropriate to their intended use and as required by the Marketing Authorisation or product specification.'[26]

There are two main components of GMP, comprising both production control and quality controls. Production control is concerned with manufacturing including the suitability of the facility and staff for manufacture, development of standard operating procedures and record keeping. Quality control is concerned with sampling, specifications and testing and with documentation and release procedures ensuring satisfactory quality.

Good laboratory practice (GLP)

The testing referred to above is conducted according to Good Laboratory Practice (GLP) which assures the quality and validity of the data generated. GLP is regulated by the Department of Health in the UK and is 'the organisational processes and the conditions under which laboratory studies are planned, performed, monitored, recorded and reported'.[27]

Compliance with GMP and GLP guidelines requires monitoring by defined quality assurance staff operating independently of personnel involved in manufacturing or testing. Clearly the implementation of such standards for early phase clinical trial material is a time-consuming and costly procedure. This is of major concern to many academic groups who are being funded to conduct research into gene therapy delivery systems for us in clinical protocols. There is, therefore, a necessity to ensure that

potential therapies based on viral vector gene delivery systems enter clinical trials in a timely and cost-effective manner without however compromising the safety and quality of the agent administered. This is a very difficult balance to achieve as the control and safety of the cell lines and of the production process for viral vector stocks is paramount for a product which is subjected to, at best, minimal purification.

There are a number of commercial service companies able to incorporate all aspects of GMP in the production of clinical grade material and which comply fully with GLP standards in the testing and control of these products. Compliance with GMP and GLP standards is the optimum for production of a medicinal product and these standards should therefore be applied whenever possible. The investment in terms of time and cost in attaining such quality standards and safety controls in a clinical protocol is a wise long-term strategy which could prevent the unforeseen and potentially disastrous contamination of a novel gene therapy agent.

Cell banking

The approach taken by manufacturers to satisfy the requirements to demonstrate cell stock identity, safety and quality involves the development of standard protocols for the selection, preparation, preservation, storage and adventitious agent testing of stock cultures for use in the production process. This approach defines what is a cell seed or virus seed lot system. Frequently the term master cell bank (MCB) or master seed stock is used to designate the stock cultures from which a second set of stock culture vials, the manufacturer's working cell bank, (WCB), is prepared. The term 'validation' describes the various assays performed on cells of the MCB and WCB to demonstrate the stability of the cells and their freedom from a variety of adventitious agents. A post-production cell bank (PPCB) consisting of cells taken beyond their normal production lifetime is produced to allow further testing for adventitious agents and genetic stability. The nature of the regulations is such that some of the recommendations and practices described here may vary.

Cell seed system for cell culture production

In the development of the cell seed system, frozen vials of a cell line are prepared at a comparatively low population doubling level to form a MCB. If large-scale production is envisaged then vials of the MCB are retrieved from storage. The cells are then grown in culture to a point predetermined by the lifespan of the cell and the number of WCB vials required. The cells are harvested and used to prepare a WCB. For initiation of production batches, a specified number of WCB vials are removed from storage and the cells placed into culture vessels with the appropriate growth medium. Scale-up of the stock cultures then proceeds according to the defined manufacturing process.

Cell banks

A master cell bank (MCB) is generated under good manufacturing practice (GMP)[26] and consists of a series of uniform vials of the cells laid down at the same time and stored in liquid nitrogen vapour.

There are a number of important considerations here:[28–31]

- During the establishment of the seed no other cell lines should be handled simultaneously in the same laboratory suite or by the same persons.
- On storage of the MCB and WCB the location, identity and inventory of individual ampoules of cells should be thoroughly documented.
- The origin, form, storage, use and details of life expectancy at the anticipated rate of use must be described in full for all seed materials. Attention should be paid to the stability of the host–vector expression system in the seed stock under conditions of storage and recovery. Any known instability should be addressed.

Preparation of the MCB under conditions of quarantine ensure the purity and identity of the stocks by preventing cross-contamination with other cell lines or adventitious agents that might be harboured by them. Continuous cell lines are commonly cloned from single cells before preparation of the MCB as an assurance of cell line purity.

Many of the observations and recommendations for preparation and storage of the MCB pertain to the WCB with the exception of clonal selection before cell bank preparation. The number of cells per WCB vial is an important assessment that should be determined within the context of the production process and requirements.

The WCB for a given manufacturing process should not be prepared until the basic parameters for the cell culture phase of the process have been elucidated. If prepared too early in process development, WCB vials may be inappropriate for use in the final production process particularly when interim changes to the culture system, medium, or inoculum density are made, which is frequently the case in experimental protocols.

Virus seed stocks

Undiluted passage of virus in cells tends to generate defective interfering particles (DIs) which is undesirable. The virus seed lot system and the use of controlled passage packaging lines should avoid the problems of a build-up of DI particles while the preparation of a virus seed pool from plaque-purified virus should eliminate or reduce DIs from these preparations.

Production of viral vectors which can replicate in a complementing cell line can also be based on a seed-lot system, such as that typically used for the production of live virus vaccines. In general, seed-lots are prepared at low passage number from a master seed lot. The master seed lot in the case of a replication-defective virus would be produced by transfecting the appropriate molecular clone into the complementing cell line and harvesting a virus stock. A working seed lot would be prepared by a single passage from the master stock.

Bacterial and plasmid seed stocks

The preparation of bacterial seed stocks under conditions of quarantine ensure the purity and identity of the stocks by preventing cross-contamination with other bacteria or adventitious agents that might be harboured by them. Description of the preparation of the master seed banks is required (including origin, form, storage and life expectancy).

The methods to preserve seed pools of *E. coli* strains use the general scheme as follows:

- Isolation of single colonies through two sequential rounds of single-colony purification as an assurance of cell line purity.

- Testing of relevant phenotypes to ensure identity.
- Growth of a suitable colony in a small-scale culture then aliquoting in small aliquots and storing in the vapour-phase of liquid nitrogen.

If possible, cells for seed pools should be grown without selective pressure. Application of selective pressure, including growth in the presence of antibiotics, introduces the risk of enriching for resistant mutants in the population of cells. If growth with antibiotics becomes necessary to select for the maintenance of unstable plasmids, an antibiotic that is not critical for subsequent use of the strain should be used. In such cases it may be useful to prepare separate master seeds of bacteria and plasmids and introduce the plasmid into the host strain before production.

Characterization and quality assurance of the seed bank and vector stocks

There are several published guidelines for the production of biological materials for use in humans (and animals).[26–34] Those dealing with recombinant DNA are particularly pertinent to the characterization and safety considerations that should be addressed when producing biological products for gene therapy.[32] There is a hierarchy of characterization and quality assurance tests that should be applied to a gene therapy viral delivery system which includes such issues as cell banking, control of production of the viral vector and quality control of the final product. The characterizations are aimed at ensuring:

- identity (cell lines/additives/viral vectors);
- reproducibility of the production process;
- freedom of substrate/products from adventitious agents and other contaminants.

Production methods for gene therapy viral vectors are essentially the same as those used for live viral vaccines where downstream processing, able to remove contaminating adventitious agents (with the exception of certain robust viruses), is minimal. Therefore, the quality of production and the level of testing conducted to detect contaminants should reflect this.

Validation of a cell should provide sufficient information to enable the manufacturer to identify the cell and to demonstrate its stability and purity. The history of a cell line/seed stock should include the origin of the cell and its passage history. Ideally, one should be able to document the original tissue from which the cell line was derived and the method of isolation. Culture conditions, media and additives (such as serum, trypsin etc) used for propagation and storage should also be described.

It is essential that the passage history of the cell be well documented. This should include documentation of the split ratio, storage conditions and all media and additives used. The source of the media and additives should be documented with suitable batch records. However, these data are frequently not available where cell lines are derived in an academic institution.

Identity

Phenotypic analysis of cell lines: A major objective in the validation of a cell line is to establish the phenotypic characteristics of the MCB. The phenotype of a cell line can then be used to monitor batch-to-batch variation during production and to verify the identity of the WCB. The methods used to establish or confirm identity often include morphology, doubling time, product expression rates, karyology, isoenzyme analysis and other markers that may be relevant within the culture process. The characterization of plasmid or gene inserts contributes to the identity of the cell line; however, this is more valuable in establishing the genetic stability of the cell line and is discussed below.

Identity is determined by a combination of cytogenetics and isoenzymes studies. Karyotypic analysis on human cell lines using Giemsa or G-banding can be very precise, yielding differences not detected by DNA fingerprinting.[35,36] However, on rodent cells it is generally a less satisfactory technique and DNA fingerprinting using minisatellite probes for hypervariable loci is particularly pertinent and may replace more traditional techniques.[36]

Virus seed stocks: A number of methods can be used to determine the identity of virus stocks including immunological markers, molecular analyses of capsid proteins or nucleic acids and phenotypic characteristics such as host range.

Bacterial seed stocks: Relevant phenotypes examined to determine the identity of a bacterial seed stock should include genetic markers that adequately validate the genetic stability of the host strain and the recombinant plasmid. Appropriate host markers may be antibiotic resistance, nutritional requirements, metabolic functions, or other traits that can be tested easily and reliably. Plasmid markers frequently include antibiotic resistance, unique for each plasmid. The identity of the host strain should be assured by phenotypic or genotypic analysis, or both, as appropriate. Lipid profiling of the host can provide useful data on cell identity and equivalency of cell stocks.

Plasmid seed stocks: If a separate plasmid seed stock is used a number of quality control tests should also be conducted. Characterization of a recombinant construct is similar to viral vectors and packaging lines including description of the assembly of the construct and the source and function of the components. Suitable restriction endonucleases will generate specific fragments that distinguish the plasmid and provide positive verification of the significant portions. The fragments should provide characteristic patterns for the coding sequence of interest and identify any potential problems of instability.

Stability of plasmids is often singled out for special attention in large-scale fermentations where segregation of cells without plasmid and maintenance of structural integrity of the plasmid are very difficult to control. Measurement of plasmid markers is used to estimate the frequency of segregation and levels of plasmid production, measured by copy number estimates, would also be relevant. Where plasmid is the end product, such features will be especially important in the absence of multiple quality control assays of final product structure.

- The expression vector has to be isolated from cells after a fermentation and digested with restriction enzymes to assure no changes have occurred throughout the fermentation.

- The nucleotide sequence of the expressed DNA has to be determined for a DNA isolated from fermentation cells, at least once in a lifetime for each master cell bank for industrial purposes.
- Other information on genetic stability, such as plasmid retention and plasmid copy number, can be gathered and it is advisable to characterize the host–vector system being used, as mentioned before.

Purity

The homogeneity and purity of the bank needs to be documented and continually monitored.

Microbiological analysis of the master cell bank is required to show freedom from adventitious agents (viruses, bacteria, fungi) and homogeneity of the culture. In the case of bacteria it is worthwhile to screen host cells for bacteriophage although lysogens can be difficult to detect, but if expressed may become a major problem during production runs.

Viability of the cells and their expression capacity should also be monitored on a routine basis. Data on the stability of the complete system including the viability of the seed stock under storage conditions is important. In addition, the method used to prepare new seed stocks needs to be described, as well as testing and acceptance criteria.

Testing for adventitious agents in mammalian cells and virus stocks

The objective in testing a cell bank for adventitious agents is to assure product safety by minimizing the potential for microbial or viral contamination. Demonstration of th purity of a cell bank includes both classic bacteria, fungi and mycoplasma testing, and also a wide range of tests designed to detect potential viral contaminations, the number of which is very large.

Contaminating micro-organisms can arise from a number of sources. These can principally be acquired from:

- The original animal used as the source of the cells including endogenous retroviruses.
- Animal products such as serum or trypsin used in the culture of the cells.
- Operators handling the cells.

While appropriate tests for bacterial and mycotic sterility must be undertaken, most problems arise from mycoplasma and viral contamination of the cell lines. The use of antibiotic-free medium imposes strict discipline on the operators and enables bacterial contamination to be detected easily. Specific testing schedules for the major screening assay systems used on cell lines have been reviewed.[2,5,37] A brief summary of an outline testing strategy is given in Table 2.

Table 2 Outline testing strategy

Testing required	MCB	WCB	PPCB
Bacteria and fungi	+	+	+
Mycoplasmas	+	+	+
General virus assays			
Electron microscopy	+	–	–
In vitro	+	+	+
In vivo	+	+	–
Retroviruses			
Reverse transcriptase assay	+	+	+
Infectivity assays for retroviruses	+	+	+
Species specific viruses			
Bovine viruses	+	–	–
Porcine viruses	+	–	–
Others	+	–	+

Genetic stability of cell line, virus vector and plasmid DNA

The characterization of the genotype of cell lines, including packaging line, viral vectors and plasmid DNA used in gene therapy is paramount[32] since the genes encoding the protein of interest are themselves the final product. As in all quality assurance testing there is a hierarchy of characterization and testing.

Characterization of a recombinant construct includes the following. Firstly, description of the assembly of the construct including the source and function of the components should be known. The complete coding sequence of the construct should be derived. In the case of master cell banks and master seed stocks of virus or plasmid DNA the complete nucleotide sequence of the gene of interest should be derived and copy number determined. A detailed restriction endonuclease map including the coding sequence should also be generated.[32,38]

Routine monitoring of the genetic stability of both the packaging/complementing cell line and virus vector during production should be attempted. Restriction endonuclease mapping is again a useful method for this. For RNA viruses a PCR step could be included before mapping. Sequence analysis of clinical lots of viral vectors may be conducted for three to five lots to ensure stability of the production system, after which restriction endonuclease mapping may be sufficient.

Genetic stability based on restriction endonuclease mapping and/or sequencing is not all encompassing and analyses of the gene products produced *in vitro*, or efficacy testing *in vivo*, will be useful parameters to ensure that the expression system is stable between production runs.

As an example of the importance of these studies it is worth noting that retroviral vectors have a propensity for mutation and recombination.[5] Similarly recombinant adenovirus type 5 vectors containing inserts giving genome sizes of 103–108% passaged in the 293 E1 complementing cell line have shown genetic instability.[39] The vector consisting of 108% of the wild-type (wt) genome replicated two- to three-fold more slowly than wild-type virus or the other vectors examined. After several passages a population of more rapidly replicating viruses was selected and viral DNA derived from this population of viruses showed a deletion of approximately 5 kb by passage 7, this deletion having been apparent in the population by passage 2. Reducing the size of the recombinant genome by 2% to 106% allowed normal replication of the vector. However, deletions were again detected at passage 4 and they were all in the insert regions. Overall, the smaller the genome the more stable the construct. These data highlight the necessity for limiting the size of the inserts, reducing passage numbers for production of adenovirus vectors and screening for genetic stability.

Plasmid stability is a signficant problem in large-scale cultures and there are four basic types of instability: segregational, insertional, structural and sequence instability. These can be detected by physical characterization of the plasmid. The gross structure and size of the plasmid in the colonies selected for phenotypic testing can be verified by electrophoretic analysis of DNA. A portion of the culture grown for preservation of a master seed pool should be removed for plasmid isolation and a restriction endonuclease map of the vector DNA sequence from the cloned gene should be confirmed. In addition, sequence of the DNA should be verified. Overall stability of the plasmid can be addressed by growing the bacteria to post-production levels, re-deriving the sequence and checking the structure by restriction endonuclease mapping.

Replication competent viruses

The basic design of viral vectors for gene therapy, cell tagging or vaccination is frequently that of a defective virus. A defective virus is defined as any virus carrying a genome lacking adequate function in one or more of the essential genes required for autonomous viral replication.[40] The majority of viruses, both eukaryotic and prokaryotic, have associated defective particles. There are five main classes of defective viral genomes that are biologically active including: integrated defective viral genomes, satellite viruses, conditionally defective genomes, pseudovirions and helper-virus dependent defective interfering particles (DIs). Transduced oncogenes carried by defective retrovirus genomes are a classic example of the hazards associated with defective viruses.

Two of the principal ways in which a defective virus can overcome the defective function are by recombination and complementation. During the construction of virus vectors one of the most important considerations with respect to the safety of the vector is the potential ability of the vector to undergo recombination with related viruses or viral sequences harboured in the genome of the cell giving rise to replication-competent recombinant viruses. An equally important consideration is the potential for the complementation of any defective functions inherent in the vector either by cellular or viral genes, leading to mobilization of the vector.

Recombination

Recombination is the physical interaction of viral genome either with viral sequences in the cell genome (eg retroviral vectors recombining with packaging sequences) or with wild-type virus which has superinfected the host (eg adenovirus vectors). Many viruses including retroviruses, adenoviruses, herpesviruses, parvoviruses and poxviruses can readily recombine with related viruses *in vitro* when there is sequence homology even when it is limited to a small region of the genome.

Complementation

In complementation there is only a functional interaction at the level of viral or cellular gene products, that is, there is no change to the genome of the defective virus vector. Currently, most defective adenovirus vectors carry a deletion in an early gene (E1a). The function of this gene can be replaced by (i) the complementation (or recombination) of this region of the E1 gene by wild-type adenovirus superinfection; (ii) cellular genes which appear to be able to transactivate in the manner of the E1 products;[41,42] (iii) the lymphotropic herpesvirus EBV which has been shown to be able to complement adenovirus E1a mutants[43] and since adenoviruses are also lymphotropic[44] there is concern that EBV would activate an E1-disabled vector.

Retroviruses

A major consideration for the safety of retrovirus vectors is the presence of replication-competent retroviruses (RCR) in the vector preparations or vector releasing packaging cell lines. Cornetta *et al*[45] has demonstrated that high levels of rcr do not have any detrimental effects when injected into non-human primates. However, in immunosuppressed monkeys several animals developed T cell lymphomas when RCR were administered along with a retroviral vector.[46] The comments below are largely directed at vectors based on MuLV; similar assay systems and methods of approach apply to other vectors, like those based on FeLV.

The murine type-C retroviruses are defined by their host range and the receptors they use to infect cells, as indicated by viral interference properties. Ecotropic viruses only infect mouse and rat cells; xenotropic viruses infect cells of many species but not murine cells. Both these classes of viruses may be inherited in the germ line as endogenous proviruses although not all murine cells express these sequences. Polytropic viruses are derived by recombination between ecotropic and other endogenous elements and can infect both murine and non-murine cells. Amphotropic viruses, used to develop vectors, were derived from exogenous viruses of wild mice and are able to infect mouse cells and cells of many other species. While the principal safety issue is the presence of amphotropic viruses, consideration should also be given to the other groups of murine retroviruses.

The presence of replication-competent amphotropic virus may be detected in an S^+L^- assay[47] which utilizes mink cells of non-transformed phenotype, harbouring a sarcoma virus genome. Other S+L- cells may be used including those of feline origin like PG4 and C81 cells. On superinfection by replication-competent virus the defective genome is mobilized by the infecting virus leading to a transformed phenotype for the cells which can be observed as a discrete focus. Under appropriate conditions, the assay is quantitative, one virus particle inducing one transformed focus. This is usually referred to as the standard or direct S^+L^- assay (Figure 2).

However, a small proportion of infectious virus does not induce a detectable focus. This is only relevant at low virus titres <10 ffu/ml and under these circumstances it is prudent to use extended assays. In this case the virus is used to infect a cell system and the cells are passaged several times to permit the virus to spread through the culture. At the end of this period the virus is assayed by S^+L^- focus formation. This assay is very sensitive but is quantal (+/–) rather than quantitative. However, it has been demonstrated that direct infection of S^+L^- cells is less sensitive for the detection of RCR than initial infection of a non-indicator line such as *Mus dunni*[48] followed by S^+L^- assay of the supernatant.[49] The most sensitive assay therefore for the detection of replication-competent retrovirus is to co-culture the packaging cell line with a cell line such as *Mus dunni* using a semi-permeable mem-

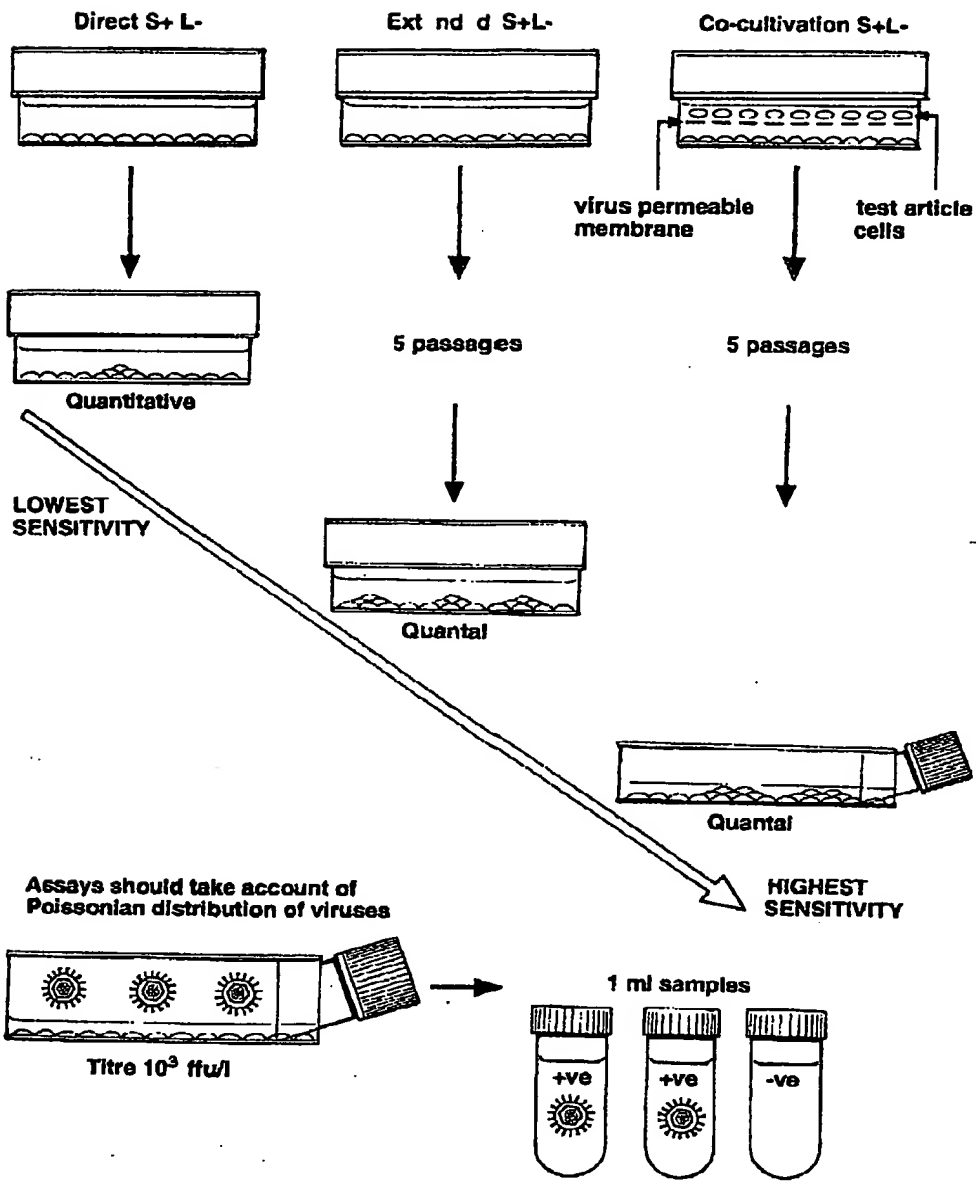

Figure 2 Sensitivity of retrovirus assays.

brane to separate the two cell types. The *Mus dunni* cells are passaged several times, then supernatant is assayed both by S^+L^- assay and by reverse transcriptase (RT) assay. Although the RT assay is less sensitive than the infectivity assay it has the advantage of being able to detect all classes of retrovirus.

Ecotropic viruses are usually detected in the XC plaque assay. Some ecotropic viruses are not easily detected in this assay and S^+L^- assays for ecotropic viruses are available. Polytropic viruses often produce a distinctive cytopathic effect on mink cells during the amplification period and some are also positive in the XC or murine S^+L^- assay.

In the first phase of evaluating clones of a vector releasing packaging line it is necessary to determine the titre of the vector. In parallel a direct *Mus dunni* assay for replication-competent amphotropic virus should also be conducted. Clones releasing high titres of the vector can then be screened more rigorously for the release of replication-competent virions. This would consist of the following:

A co-cultivation assay with three cell types is initiated: (1) A murine cell line like SC-1 or FG10 that is permissive for all ecotropic type C murine viruses. (2) A *Mus dunni* cell line that is permissive for xenotropic and amphotropic viruses. (3) A human diploid cell line. These are permissive for amphotropic viruses and for some xenotropic viruses. In our experience xenotropic viruses derived from murine hybridoma lines often fail to repli-

cate efficiently in human cells. However it is an FDA requirement that when cells used in the manufacture of biological products have evidence of retrovirus production then the ability of these viruses to replicate on human cells should be determined.

At the end of the passage period these cells are examined by at least two independent criteria for the release of replication-competent retroviruses such as S^+L^- and reverse transcriptase assay described previously.

After the clinical lot has been produced it is also necessary to produce a post-production cell bank and to repeat the tests to validate that long-term culture does not lead to recombination and the generation of wild-type virus.

Adenoviruses

Retrovirus vectors are produced continually by a transfected packaging line and it is therefore possible to screen both the producer cell line and the vector stock for RCR.

Adenovirus vectors, however, are produced by transfection of DNA followed by production of replication-defective infectious virus which can only replicate in the complementing cell line. This is also true for certain herpesvirus and parvovirus vector constructs.

The presence of RCV in adenovirus vector stocks can be detected by amplification on adenovirus-susceptible cell lines and by polymerase chain reaction (PCR) amplification across deleted regions of the virus genome. The high titres of virus used in adenovirus vector gene therapy protocols requires sensitive screening for RCVs to detect one or two RCVs in 10^{12} virus vectors. Typical testing for RCV adenovirus would include passaging of vector stocks to amplify replication competent viruses in permissive cells such as HeLa. The high titre stocks of adenovirus vectors may make such testing problematic and a custom PCR assay for unintentional recombinant viruses may be required.

Future development of complementing cell lines and improvements in the design of the basic vector spine should reduce the potential for the production of rcv.[4]

Herpes virus

As in all virus vector stocks the detection of replication competent viruses is paramount and can be addressed by *in vitro* and *in vivo* assays.

In addition, recombination of herpes vector sequences with herpes viruses present in patients either as replicative or latent infections is possible. Herpes viruses readily recombine *in vivo*[50] and the generation of replication competent or virulent recombinant virus in a patient is of concern.

Herpesvirus RCV can be detected by passaging the virus vector stock on BHK cells to amplify any RCV and examining the culture for the formation of syncitia or plaques of cell death. This assay can only be applied to vectors with deletions in non-essential regions and *in vivo* testing may be required to detect any revertant virus.

There are several *in vivo* model systems to address the problems of pathogenicity, latency, gene expression, recombination and reactivation of herpesvirus mutants and vectors.[51,52] The models are based on the ability of herpesviruses to replicate in the animal systems and establish latency and include footpad inoculation or corneal infection of mice. Data derived from the use of such animal models will give useful information on the characteristics and pathogenicity of herpesvirus vectors. However, each vector will require a customised protocol to address particular safety considerations.

A robust *in vitro* model for latency studies would be particularly useful in the characterization of recombinant herpesviruses.

Production and purification

The production of viral vector stocks involves little downstream purification and is akin to production of live viral vaccines. It is inevitable that gene-therapy vectors will contain DI particles and in many cases effective assays for DIs are not available. It is possible in some cases to purify DI particles from virus preparations by velocity gradient centrifugation, however, this is again impractical or impossible for most vector preparations.

Adventitious agent testing of vector stocks

In addition to the test for RCVs described for viral vector stocks, similar tests to those described for the cell banks, ie identity, sterility, mycoplasma and adventitious agent tests are also required for vector stocks. The importance of testing cell banks and vector stocks for use in gene therapy application cannot be overemphasized as the lack of downstream purification (dsp) removes one of the major contributions to the safety of these products with respect to contamination with adventitious agents. Such a lack of dsp also has relevance to the level of impurities which may occur in these products.

As previously mentioned, during production of a 'normal' biopharmaceutical product there is fairly extensive downstream processing and the purity and integrity of the product and monitoring of impurities are important quality control parameters. Impurities such as host cell proteins, contaminating proteins from FCS and additives, contaminating DNA, endotoxin and other relevant molecules are measured and permissible limits set. Analyses of viral vectors in terms of a biopharmaceutical protein are nearly always impossible since purification of vectors is minimal. Indeed, crude cell supernatants may be administered directly to patients. Particularly in the case of multiple dosing it would be worthwhile if cells could be adapted to serum-free media with a minimum of additives to reduce levels of contaminating FCS and other immunologically active proteins. In addition increasing viral titres would lower the volume of doses required to be administered to patients.

As for mammalian cell production, so bacterial cultures need to be routinely monitored for microbial contamination. Sterility checks should be done on the inoculation vessel, fermenter, and the fermentation medium. The process should also be monitored for adventitious agents by screening the fermentation for infection by unwanted bacteria, fungi or bacteriophages. The presence of contaminating organisms will alter the levels of production and thereby contribute to the levels of contaminating impurities within the final DNA product. The contents of any fermenters containing bacterial, fungal or viral contamination should be discarded.

Impurities

Impurities in vectors for gene therapy are of concern and limits on the levels of such impurities must be set. The main contaminating impurities which should be addressed are discussed below:

Host cell proteins: Multiple injections of a vector require the quantification of host cell contaminating proteins, eg *E. coli* proteins or proteins from the culture medium since they may cause problems of immunogenicity.

A control run of plasmid purification would normally be performed with the host cell alone and the material collected at the expected stage. This material would be used to immunize rabbits, thereby facilitating the development of an ELISA or similar assay to determine levels of contaminating proteins in the bulk plasmid.

Contaminating DNA: DNA present in final products can be considered to be potentially hazardous and there are limits set on the levels of contaminating DNA in biologicals derived from transformed eukaryotic cells. Contaminating DNA from eukaryotic cells contains activated oncogenes which are not a major consideration with prokaryotic production systems. However, monitoring of DNA levels in final product should be considered even if only as a quality control measure.

The most effective means established for determining the removal of nucleic acids in a process involves running host cells alone through the process and estimating the levels of contaminating DNA expected in each batch of plasmid. In addition, spiking DNA fragments into impure product obtained from the fermenters and noting the amount of reduction in DNA that occurs during subsequent processing steps can be performed. By this procedure it is possible to estimate how much nucleic acid material could be carried through the process in a 'worst-case' scenario. These data should be sufficient to replace the determination of contaminating host cell DNA in final product although routine monitoring is still useful.

It may be preferable that any antibiotic resistance genes be removed from the plasmid before administration to patients. This would require the development of procedures to purify the DNA of interest and the validation of removal of the genes. Measurement of residual antibiotic resistance gene DNA in the final product can then be undertaken.

Endotoxin: Endotoxins are derived from the lipopolysaccharide entities of the Gram-negative host organisms used in the production of the DNA. Such contamination can be measured and rabbit pyrogen testing and LAL assays are essential for DNA products and viral vector stocks. It may also be necessary to conduct an endotoxin validation on the purification process.

In vivo or in vitro functional assay: Inoculation of a known amount of the final product into mice and guinea pigs can be used to monitor any abnormal toxic reactions. However, an important part of QC testing for DNA products and indeed virus vectors is an *in vitro* functional assay which gives details of the expression and product profile from each plasmid batch. A carefully designed assay could, in certain cases, allow some analysis of the final product expressed by the vector or plasmid DNA similar to those performed routinely on other biologicals.

Ligands: The requirements to determine the safety, consistency and quality of DNA products will of course apply to any agent administered with the DNA. Consideration should be given to such agents including liposomes, cell targeting moieties, and virus capsids linked to the DNA.

Patient monitoring

Careful monitoring of patients involved in clinical trials provides useful data on the efficacy of a gene therapy agent. However, other considerations are important in selecting patients for a trial and for continued safety screening. The presence of integrated/defective viral genomes in either patient or packaging/complementing cell line could result in recombination with the vector and lead to infectious virus or pseudotyping. It is generally impractical to screen patients for defective genomes and it should be assumed that all patients will harbour them. Suitable precautions and even screening (eg ELISA) may be prudent for replication competent herpes/adenovirus. In addition, screening of patients for recombinant replication-competent virus that may be generated after administration of a vector provides additional data for safety considerations of particular vectors.

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